International Journal of Molecular and Clinical Microbiology 1 (2013) 244-247

International Journal of
Molecular and Clinical Microbiology

Evaluation of biofilm-forming capabilities of urinary *Escherichia coli* isolates in microtiter plate using two different culture media

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ARTICLE INFO

Article history:
Received: 21 December 2012
Accepted: 13 March 2013
Available online: 1 June 2013

Keywords:
biofilm, uropathogenic *E. coli* (UPEC), LB medium, BHI broth

ABSTRACT

The ability of uropathogenic *E. coli* (UPEC) to cause symptomatic UTI is associated with the expression of a variety of virulence factors. Biofilm formation enables UPEC to resist the flow of urine and increases its tolerance to antimicrobials and the host immune response. The measurement of biofilm formation in vitro is affected by the type of culture medium used. The aim of this study was to evaluate biofilm-formation capabilities of UPEC in microtiter plate using two different culture media. A total of 170 isolates of *E. coli* were isolated from patients with symptomatic UTI in Gorgan, north of Iran. Biofilm formation of the strains was examined in LB and BHI broth with the addition of 1% sucrose. The quantitative analysis of biofilm formation was performed using crystal violet staining followed by spectrophotometry measurement after addition of decoloring solution. The biofilm formation of UPEC isolates in LB broth (20 isolates; 11.8%) was significantly ($p < 0.001$) lower than those grown in BHI broth (105 isolates; 61.8%). All isolates that formed biofilm in LB broth also formed biofilm in BHI broth. Whilst 36 (21.2%) isolates grown in BHI broth formed strong biofilm, only one (0.6%) isolate grown in LB broth exhibited a similar result ($P<0.007$). Our data suggest that the process of biofilm formation by UPEC is strongly modulated by culture conditions and the method employed. In our study the use of BHI broth supplemented with 1% sucrose proved to be superior to the LB broth and can be employed for measurement of biofilm formation in UPEC.

1. Introduction

Biofilms are collection of microbial cells that are irreversibly associated with surfaces and cannot be removed by gentle rinsing. This structures are enclosed in a matrix of primarily polysaccharide material allowing growth and survival in sessile environment(Hall-Stoodley *et al.*, 2004). In some cases, clusters of cells are separated by channels through which fluid can move. As the bacterial cells adapt to growth in these structures, they express new phenotypic traits that are often distinct from those that are expressed during planktonic growth (Costerton *et al.*, 1999). These properties include increased resistance to antibiotics, drought, radiation, toxins, changes in pH, temperature fluctuations and washing or hydrodynamic pressure in body fluids such as urine flow (Sadashivaiah & Mysore, 2010). Biofilm growth of pathogenic bacteria on tissues or indwelling devices often results in infections that increase tolerance to antimicrobials and the host immune response. The antimicrobial resistance mechanism of biofilm is

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probably multifactorial. Examination of the physiology of biofilm-associated bacteria is necessary for understanding infections as well as other processes that are mediated by microorganisms(Stewart & Franklin, 2008). Biofilms formed by bacterial pathogens on medically relevant surfaces are difficult to eradicate and are thus often involved in the development of infections(Da Re *et al.*, 2007). Urinary tract infections (UTI) are one of the most common bacterial infections in humans. *Escherichia coli* is responsible for more than 80% of all UTIs (Hedlund *et al.*, 2001). Biofilm formation has an important role in the pathogenesis of uropathogenic *E. coli* (UPEC)(Soto *et al.*, 2007). Identification of *E. coli* strains capable of producing biofilms is useful in understanding the pathogenesis of this bacterium in development of UTI. Currently, there are different in vitro methods of assessing biofilm formation such as coverslip assay, scanning electron microscope, Congo red agar method, flow cell and impedance method as well as assessing the formation of biofilm in animal models (Hachem *et al.*, 2009; Marhova *et al.*, 2010; Meshram *et al.*, 2012; Solano *et al.*, 2002; Watnick *et al.*, 1999; Zikmund *et al.*, 2010). One of the most common quantitative methods is microtiter plate assay, which is widely used in different studies (Costerton *et al.*, 1999; Marhova *et al.*, 2010; Meshram *et al.*, 2012).

In this method, bacterial suspension are grown in culture media such as LB and TSB broth and then are transferred into wells of a microtiter plate and the formation of biofilm is evaluated after 24 hours. However, it has been suggested that the biofilm formation of bacteria is partly depended on the type of medium and the assessment methods (Meshram *et al.*, 2012; Naves *et al.*, 2008). The aim of this study was to compare the ability of two growth media on biofilm-formation of a collection of uropathogenic *E. coli* isolates.

2. Matherials and Methods

2.1. Bacterial strains

A total of 170 isolates of *E. coli* were collected in Gorgan - north of Iran - from Urine samples of hospitalized and non-hospitalized patients during the years 2010-2011. Strains were confirmed as *E.coli* using conventional methods and were cultured in TSB broth for 24 h at 37 °C.

2.2 Biofilm formation assay

Quantification of biofilm formation of the isolates was done using the microtiter plate method of Meshram et al. (Meshram *et al.*, 2012) and Watnick et al. (Watnick *et al.*, 1999) with slight modifications.

In the first method, 30 µl of each overnight culture was transferred to 3 ml of sterile LB broth for production of biofilm. 200 µl of a bacterial suspension, corresponding to McFarland tube 0.5 (absorbance value of 0.08-0.1 at 625 nm) were transferred into wells of microtiter plate and inoculated at 37°C for 24 hours without shaking. Wells with 200 µl of only LB broth was considered as negative control (Marhova *et al.*, 2010; Watnick *et al.*, 1999). In the second method, 30 µl of each overnight culture was transferred to 3 ml of sterile BHI broth containing 1% (w/v) sucrose. The suspension was adjusted to approximately 10^2 CFU/ml and 200 µl was transferred into wells of a microtiter plate and incubated at 37°C for 24 hours without shaking. 200 µl of only BHI broth containing 1% (w/v) sucrose was used as negative control (Meshram *et al.*, 2012).

E. coli 1399 PTCC was used as a positive control in both methods. All experiments were performed triplicate.

2.3. Evaluation of biofilm formation

After 24 hours, the planktonic suspension and nutrient solution were aspirated and each well was washed three times with 300 µl of sterile physiological saline. Each well was then stained for 5 min using 200 µl of 2% crystal violet (CV). The excess CV dye was removed and this was followed by washing the microtiter plates three times with PBS. To release the CV, 200 µl of ethanol/acetone (80:20) was added to the wells and after 15 min the absorbance was measured at 600 nm to estimate the amount of biofilm formed.

A similar methodology was used for samples inoculated in BHI containing 1% (w/v) sucrose and the suspension was washed three times with 300µl of sterile physiological saline. The plates were strongly shaken in order to remove all non-adherent bacteria. The remaining attached bacteria were fixed with 250 µl of 96% ethanol per well for 15 min after which the plates were dried. Each well was then

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stained for 5 min. with 200µl of 2% CV. The plates were then washed under running tap water. The quantitative analysis of biofilm formation was performed by adding 200µl of 33% (v/v) glacial acetic acid per well. The optical density (OD) of the stain was measured at 492 nm using an ELISA reader.

Biofilm formation was scored as follows:

Biofilm formation OD = stained attached bacteria OD – stained control wells OD

OD≥0.3 was regarded as strong biofilm formation whilst OD between 0.299 and 0.2 was scored as moderate biofilm formation and finally an OD between 0.199 and 0.1 was regarded as weak biofilm formation. All OD≤0.1 were regarded as no biofilm formation(Naves *et al.*, 2008).

3. Result

In this study, 170 bacterial isolates were obtained from urine samples of 59 hospitalized and 111 non-hospitalized patients. In the first method (LB medium), a total of 20 isolates (11.8%) were considered positive for biofilm formation but in the second method (BHI containing 1% (w/v) sucrose), 105 isolates (61.8%) formed biofilm. This difference was statistically significant (P<0.001). All isolates that formed biofilm in LB medium also formed biofilm in BHI medium (Figure 1).

In the first method, only one isolate (0.6%) had a strong biofilm, one isolate (0.6%) had a moderate biofilm and 18 isolates (10.6%) showed weak biofilm formation. In the second method, 36 isolates (21.2%) showed strong biofilms, 17 isolates (10%) showed moderate biofilms and finally 52 isolates (30.6%) demonstrated weak biofilm formation (P<0.007) (Figure 1).

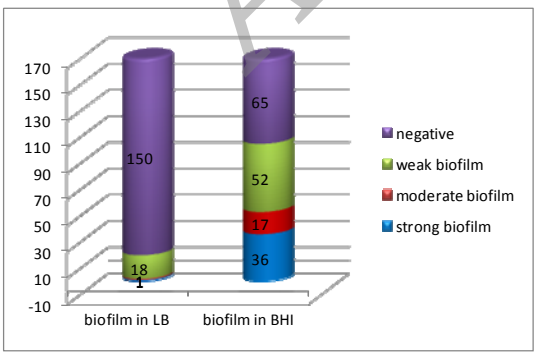

Figure 1. Comparison of biofilms formed by uropathogenic *E. coli* in two different culture media.

4. Discussion

Quantitative microtiter plate assay is a widely used method in different studies (Abdallah *et al.*;2010; Marhova *et al.*, 2010; Reisner *et al.*, 2006; Schembri & Klemm, 2001; Soto *et al.*, 2007). In many studies, Luria- Bertani broth (LB) has been used for detecting biofilm formation in microtiter plate method. Barrios et al. (2006) studied biofilm formation of a collection of *E. coli* in LB, M9C and LB containing glucose and showed that 74% , 46% and 78 % of isolates can form biofilm, respectively (Barrios *et al.*, 2006). Similarly Schembri et al. (2001) showed that 60% of their *E. coli* isolates can produce biofilm in LB broth (Schembri & Klemm, 2001). Contrary to these findings, in a study by Naves et al. (2008) on biofilm formation of *E. coli* in the LB, M63, M9 and MHII (Mueller–Hinton II broth) it was shown that biofilm formation is higher in MH-II and M9 and lower in LB and M63 media.

E. coli ATCC 25922 could form biofilm in a rich medium (MH-II) but did not form biofilm in LB broth (Naves *et al.*, 2008). Another contradictory results reported by Ferrières and co-workers (2007) on biofilm-forming capacities of a set of UTI *E. coli* strains encompassing 11 ABU (asymptomatic bacteriuria) strains and six UPEC isolates in the LB broth with human urine indicated that 72% of the ABU strains formed biofilm while UPEC strain lacked the capability of forming biofilm (Abdallah *et al.*, 2010). However, in this study, only 20 % of 170 *E. coli* isolates were able to form biofilms in LB medium. On the other hand, considering the importance of biofilm formation by UPEC, it seems that these results were an underestimation of the actual biofilm-forming ability of these strains. Therefore, in the present study, another method with BHI medium containing 1% sucrose was used. In a study by the Meshramet al. (2012) on *E.coli* biofilm formation in BHI broth containing 1% sucrose, it was indicated that 76 % of isolates can form biofilm (Meshram *et al.*, 2012).

Another study by Stepanovic et al. (2004) on biofilm-forming capacities of *Listeria monocytogenes* in the BHI, TSB and MB (meat broth) showed that the ability to form biofilms in BHI medium was much higher (83.3%) than that of TSB (14.6%) and MB (2.1%) (Stepanović *et al.*, 2004). Contradictory to this study, Skyberg et al. (2007) found that only 29.4% *E.coli* can form

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biofilm in this medium and 44.7% of them produced biofilm in TSB (Skyberg *et al.*, 2007). In our study, the ability of bacteria to form biofilms was 61% with this method. All isolates that formed biofilm in LB medium, could also form biofilm in BHI medium. Furthermore, a number of strains that did not form biofilm in LB medium had the ability to form biofilms in BHI. One of the possible reasons for the observed increase in biofilm formation in BHI is fixing the biofilm by addition of the ethanol, which could prevent the possible removal of biofilms during staining.

Conclusion

Our data indicates that the process of biofilm formation by *E. coli* depends on the strain properties, and it is strongly modulated by culture conditions, environmental factors and methodology.

Acknowledgements

We thank the staff of Golestan University of Medical Sciences, Maya Babai and Hanie Bagheri for providing isolates and Masood Bazori for logistical support.

This work was supported by a grant, awarded to Infectious Disease Research Center and Technology and Research Deputy of Golestan University of Medical Sciences (contract no.35/2526).

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